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Original Article

# *IN VITRO* ANTIOXIDANT PROPERTY OF FRACTIONS OF *ANNONA RETICULATA* (L) LEAVE

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**ABSTRACT**

**Objective:** The present study has been carried out to evaluate the antioxidant property of different fractions prepared from hydro-alcoholic extract of *Annona reticulata* L. leave. Effort also has been made to estimate the flavonoid and phenolics content of the fractions.

**Method:** The antioxidant activity has been studied *in vitro* by using Nitric oxide-scavenging assay, Free radical scavenging activity in DPPH assay and Iron chelating activity assay.

**Results:** In Nitric oxide-scavenging assay the $IC_{50}$ values for Ethylacetate fraction, methanol fraction and residue/aqueous fraction are 476.43 µg/mL, 328.09 µg/mL and 329.61 µg/mL respectively. In DPPH assay the $IC_{50}$ values for Ethylacetate fraction, methanol fraction and residue/aqueous fraction are 87.50 µg/mL, 161.06 µg/mL and 90.36 µg/mLrespectively. The $IC_{50}$ values of the Ethylacetate fraction, methanol fraction and residue/aqueous fraction in Iron chelating assay are 210.86 µg/mL, 462.38 µg/mL and 586.14 µg/mL respectively. The flavonoid content of Ethylacetate fraction, methanol fraction and residue/aqueous fraction are 8.73, 7.45 and 8.62 respectively expressed as mg/g of Catechol. Similarly the phenol content of Ethylacetate fraction, methanol fraction and residue/aqueous fraction are 8.43, 7.25 and 6.23 respectively expressed as mg/g of Catechol.

**Conclusion:** The results suggest that all the tested fractions are having antioxidant property, but the ethhylacetate fraction is having significantly higher flavonoid and phenol content. Due to presence of higher flavonoid and phenol content in ethylacetate fraction, it may be considered as the fraction with better pharmacological property in comparison to other tested fractions.

**Keywords:** *Annona reticulata*, Antioxidant, DPPH, Iron Chelating.

## INTRODUCTION

*Annona reticulata*Linn. (Family – *Annonaceae*) commonly known as bullock's heart or raamphal plant, is widely distributed all over India and tropical parts of the globe. In traditional medicinal system different parts of the plant are used as insecticides, anthelmintic, styptic, suppurant, astringent, antidysentric and vermifuge. Root bark, leaves and stem possess isoquinoline alkaloids [1]. Previous experiment conducted by us revealed that, the hydro-alcoholic extract of leave of *Annona reticulata* Linn. Is having potential anti-hyperglycemic property in streptozotocin induced animal models [2]. Oxidative stress is known to play major role in development of diabetes and its related complications as well as several other several metabolic disorders, and use of antioxidants may be considered as one of the approaches for management of those disorders [3, 4]. The presented experiments were carried to evaluate the antioxidant potential of different fractions prepared from hydro-alcoholic extract of *Annona reticulata*Linn. leave *in vitro*.

## MATERIALS AND METHODS

### Preparation of fractions

The leave of *Annona reticulata* L. (Family – Annonaceae) were collected in the month of July – August 2010, from the rural area of the dist. Cuttack, Odisha, India and after the plant has been authenticated by Taxonomist from Central Rice Research Institute, Cuttack, Odisha, India. Course powder of dried leaves was prepared and initially defatted with petroleum ether followed by 72-hours extraction with 1: 1 mixture of methanol and water using cold maceration process for 72-hours to get the hydro-alcoholic extract. The dried extract was used for preparation of different fractions by using solvents with solvents in increasing order of polarity by following earlier published methods [5, 6, 7]. The method of fractionation can be summarized as follows; 20 gms of hydro-alcoholic extract of *Annona reticulata* leave was taken in a separating funnel and dissolved in 50 ml of distilled water. To the solution 50 ml of Chloroform was added and then shaken vigorously. The chloroform layer was then collected by filtration and dried by using the rotary evaporator. To the left over layer 50 ml of Ethyl acetate was added by shaking and ethyl acetate layer was separated and dried to get Ethyl acetate fraction. To the left over fraction 50 ml of methanol was added and shaken to get the methanol soluble substance and Methanol fraction is prepared by drying the filtered solution. The remaining layer or filtrate was collected and evaporated to get the Residual fraction or the aqueous fraction. For the purpose of filtration Whatman filter paper, No 3 was used. The percentage yields of the fractions with respect to dried extract are; Chloroform fraction -1.00% w/w, Ethyl acetate fraction - 15.50 % w/w, Methanol fraction - 12.80% w/wand Aqueous fraction or Hydro-alcoholic fraction or Residue - 25.50 % w/w. Out of the four fraction three fractions i. e. Ethyl acetate fraction, Methanol fraction and the residue or aqueous fraction had been taken for further studies. The chloroform fraction has not been taken for experimental purpose as the yield was not sufficient enough to carry out the planned studies.

### Assay of nitric oxide-scavenging activity

The procedure was based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using spectrophotometer in presence of Griess reagent [8]. For the experiment, sodium nitroprusside (5 mM), in phosphate-buffered saline (0.025m, pH 7.4), was mixed with different concentrations of each fraction (125 - 1000 µg/ml) as well as standard Ascorbate dissolved in water and incubated at room temperature ($29^0$C) for 180 min. Following the incubation period, 1 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 550 nm and ascorbate was used as a standard. The % activity was determined by the formula [8];

% inhibition = [(Absorption of Control – Absorption of Test) / Absorption of Control] X 100

Log (Concentration) vs. % Activity graph has been plotted to determine the $IC_{50}$ value and compared to standard. The results have been depicted in table 1 and fig. 1 of results section.

**Free radical scavenging activity in DPPH assay**

The radical scavenging activity of the fractions against stable DPPH* was determined by spectrophotometry method as explained by Brand-Williams*et al.*[9] with a slight modification to it. The basic fundamental of measuring antioxidant efficiency in DPPH* free radical method is to eliminate the risk of thermal degradation of the molecules tested by measuring the activity at ambient temperature [10]. DPPH is a stable free radical and accept an electron, or hydrogen radical to become a stable diamagnetic molecule. DPPH reacts with an antioxidant compound that can donate hydrogen & get reduced. The change in colour (from deep violet to light yellow) was measured. The intensity of the yellow colour depends on the amount and nature of radical scavenger present. The reaction mixture was prepared by mixing of 1 mL of DPPH solution, 1 mL of methanolic solution of various concentration of the fractions (125, 250, 500, and 1000 µg/mL) and the volume was made up to 3 mL with water. The tubes were incubated for 15 minutes at room temperature in dark and decrease in absorbance was measured at 517 nm. Rutin was used as standards [11].

Radical scavenging activity was calculated by using the formula [9];

$$\% \text{ Activity} = [(A_C - A_T)/A_C] \times 100$$

Where $A_C$ = absorption of blank sample (t= 0 min),

$A_T$ = absorption of test solution (t=15 mins)

Log (Concentration) vs. % Activity graph has been plotted to determine the $IC_{50}$ value and compared to standard and the results have been presented in table 2 and Figure2.

**Iron chelating activity assay**

The method published by Sathish *et al*. was followed for Iron chelating activity assay [12]. The test is based on the principle of formation of *O*-Phenanthroline-$Fe^{2+}$ complex and its disruption in the presence of chelating agents. The reaction mixture was prepared by mixing 1 mL of 0.05% *O*-Phenanthroline in methanol, 2 mL Ferric chloride (200µM) and 2 mL of various concentrations of the fractions ranging from 125 to 1000µg. The reaction mixture was incubated at room temperature for 10 min and the absorbance was measured at 510 nm. EDTA was used as standard metal chelating agent. The percentage activity of each sample was calculated by using the formula [13]:

% inhibition = [(Absorbance of Control – Absorbance of Sample)/ Absorbance of Control] × 100.

Log (Concentration) vs. % Activity graph has been plotted to determine the $IC_{50}$ value and compared to standard and mentioned in table 3 and fig. 3.

**Total flavonoids content determination**

The method published by Jaslin and Padmaja was followed for determination of total flavonoid content in the fractions [14]. 200mg of the fraction(s) was ground with a mixture of ethanol and water in 2 different ratios (9:1 and 1:1 respectively). The homogenate mixtures formed were filtered and were combined. The final mixture formed was dried to remove ethanol by the process of evaporation and the resultant aqueous solution was extracted in a separating funnel with hexane or chloroform.

The resulted aqueous layer was concentrated and out of that an aliquot of 0.5 ml was pipette-out in a test tube. To the test tube 4 ml of the vanillin reagent (1% vanillin in 70% conc. $H_2SO_4$) was added and kept in a boiling water bath for 15 minutes, cooled and then the absorbance was taken at 360 nm by using spectrophotometer. A standard was run by using catechol (5-25µg/mL). The flavonoid content was calculated from a catechol standard curve and expressed in mg catechol equivalent per gm of dry weight [15]. The total flavonoid content of the fractions has been presented in table 4 and in fig. 4 as well.

**Total phenol content determination**

The measurement of total phenol has been done as per the method described by Khatiwora*et al*. [16]. 250mg of sample was taken in a 10 mL test tube and 2.5 ml of ethanol was added to it and centrifuged at 2°C for 10 minutes. The supernatant was preserved. Then, the sample was re-extracted with 2.5 ml of 80% ethanol and centrifuged. The pooled supernatant was evaporated to dryness. Then to the dried supernatant water was added to make the volume 3 mL. To the solution 0.5 ml of Folins phenol reagent (Folinciocalteau reagent; 1:1 with water) and 2 mL of Sodium Carbonate (20%) was added sequentially. A blue coloured complex (Molybdenum blue colour) was developed in the test tube, as the phenols undergo a complex redox reaction with phosphomolibdic acid present in folinciocalteau reagent in alkaline medium. The reaction mixture was kept on boiling water bath for 1 minute, cooled and the absorbance was measured at 650 nm in a spectrophotometer. Standard calibration curve was plotted generated at 650 nm using known concentrations of catechol (5-25 µg/mL) and concentrations of phenols in the test samples were calculated from the calibration plot and expressed as mg catechol equivalent of phenol/g of sample. The values for total phenolic content of the fractions have been represented in table 5 and fig. 5.

**Statistical analysis**

Results are expressed as Mean ± SE. The data were analyzed by one way ANOVA followed by Turkey – Kramer Multiple Comparison Test. Confidence interval has been considered as 95% and $p < 0.05$ were considered significant.

**RESULTS**

The data generated from the Nitric Oxide free radical scavenging activity assay of the fractions (Table 1, fig. 1), it is evident that all the fractions are having potential NO free radical scavenging activity. The $IC_{50}$ values of the Ethylacetate fraction, Methanol fraction and Residual fraction are 476.43 µg/ml, 328.09 µg/ml and 329.61 µg/ml respectively. The $IC_{50}$ value of standard Ascorbate is 387.26 µg/ml. Among the three fractions methanol fraction is having the lowest $IC_{50}$ value (328.09 µg/ml) in the case of NO scavenging assay.

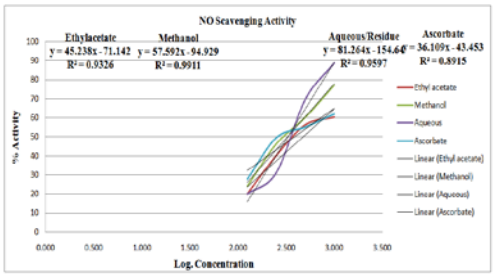


**Fig. 1: Nitric oxide free radical scavenging activity of Fractions**

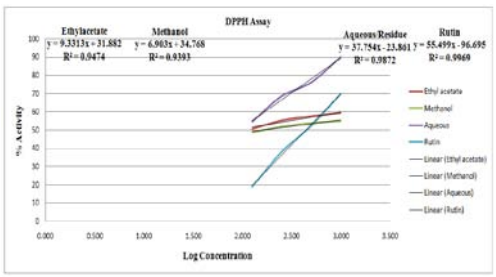


**Fig. 2: Free radical scavenging activity of Fractions in DPPH Assay**

The free radical scavenging activity of the fractions in DPPH Assay model has been explained in table 2 and fig. 2. The results of the

study shows that the $IC_{50}$ values for free radical scavenging in DPPH model for Ethylacetate fraction, Methanol fraction and residual fraction are 87.50 µg/ml, 161.06 µg/ml and 90.36 µg/ml respectively. The $IC_{50}$ value of standard Rutin is 39.54 µg/ml. Among the three fractions ethylacetate is having the lowest $IC_{50}$ value(87.50 µg/ml) in the case of DPPH free radical scavenging assay.

**Table 1: Nitric oxide free radical scavenging activity of fractions**

| S. No. | Concentration (µg/ml) | Percentage activity | | | |
|---|---|---|---|---|---|
| | | Ethylacetate fraction | Methanol fraction | Residue fraction/Residue fraction | Standard(Ascorbate) |
| 1 | 125 | 20.12±0.09 | 23.95±0.02[a,d] | 20.12±0.03 | 27.63±0.03[c,e,f] |
| 2 | 250 | 40.14±0.04[b] | 46.2±0.02[a,b,c] | 31.20±0.01 | 49.53±0.01[c,d,e] |
| 3 | 500 | 56.14±0.05[c] | 60.12±0.01[a,e] | 69.70±0.01[b,d,f] | 55.12±0.01 |
| 4 | 1000 | 60.18±0.02 | 77.1±0.01[a,e] | 88.83±0.02[b,d,f] | 62.00±0.04[c] |
| $IC_{50}$µg/ml | | 476.43 | 328.09 | 329.61 | 387.26 |

Values are expressed as Mean ± SEM; (n = 3); Different Superscripted letters (a, b, c,d, e and f) means in the same row Means are significantly different at P < 0.05 (One Way ANOVA followed by Turkey – Kramer Multiple Comparison test); [a]-Comparison between Ethyl acetate group and Methanol Group of same row; [b]-Comparison between Ethyl acetate group and Residue Group of same row; [c]-Comparison between Ethyl acetate group and Standard Group of same row; [d]-Comparison between Methanol group and Residue Group of same row; [e]-Comparison between Methanol group and Standard Group of same row; [f]-Comparison between Residue group and Standard Group of same row

**Table 2: Free radical scavenging activity of fractions in DPPH assay**

| S. No. | Concentration (µg/ml) | Percentage activity | | | |
|---|---|---|---|---|---|
| | | Ethylacetate fraction | Methanol fraction | Aqueous fraction/Residue fraction | Standard (Rutin) |
| 1 | 125 | 50.65±0.02[a,c] | 48.64±0.02[e] | 54.63±0.02[b,d,f] | 18.85±0.05 |
| 2 | 250 | 55.22±0.02[a,c] | 51.95±0.04[e] | 68.64±0.03[b,d,f] | 38.08±0.01 |
| 3 | 500 | 57.54±0.01[a,c] | 53.95±0.04[e] | 76.12±0.01[b,d,f] | 52.21±0.02 |
| 4 | 1000 | 59.24±0.02[a] | 54.90±0.02 | 90.02±0.02[b,d,f] | 69.83±0.02[c,e] |
| $IC_{50}$ µg/ml | | 87.50 | 161.06 | 90.36 | 39.54 |

Values are expressed as Mean ± SEM; (n = 3); Different Superscripted letters (a, b, c, d, e and f) represents; in the same row Means are significantly different at P < 0.05 (One Way ANOVA followed by Turkey – Kramer Multiple Comparison test); [a]-Comparison between Ethyl acetate group and Methanol Group of same row; [b]-Comparison between Ethyl acetate group and Residue Group of same row; [c]-Comparison between Ethyl acetate group and Standard Group of same row; [d]-Comparison between Methanol group and Residue Group of same row; [e]-Comparison between Methanol group and Standard Group of same row; [f]-Comparison between Residue group and Standard Group of same row

**Table 3: Iron-chelating activity of fractions**

| S. No. | Concentration (µg/ml) | Percentage activity | | | |
|---|---|---|---|---|---|
| | | Ethylacetate fraction | Methanol fraction | Aqueous fraction/Residue fraction | Standard(EDTA) |
| 1 | 125 | 39.20±0.01[a,b] | 21.50±0.09[b] | 22.80±0.03[a,d] | 58.68±0.04[c,e,f] |
| 2 | 250 | 51.90±0.01[a,b] | 34.90±0.02 | 36.10±0.01[d] | 65.87±0.01[c,e,f] |
| 3 | 500 | 69.80±0.01[a,b] | 51.70±0.02[d] | 46.90±0.02 | 83.83±0.03[c,e,f] |
| 4 | 1000 | 89.10±0.02[a,b] | 67.80±0.04[d,e] | 59.20±0.04 | 97.90±0.02[c,f] |
| $IC_{50}$ µg/ml | | 210.86 | 462.38 | 586.14 | 90.99 |

Values are expressed as Mean ± SEM; (n = 3); Different Superscripted letters (a, b, c,d, e and f) means in the same row Means are significantly different at P < 0.05 (One Way ANOVA followed by Turkey – Kramer Multiple Comparison test); [a]-Comparison between Ethyl acetate group and Methanol Group of same row; [b]-Comparison between Ethyl acetate group and Residue Group of same row; [c]-Comparison between Ethyl acetate group and Standard Group of same row; [d]-Comparison between Methanol group and Residue Group of same row; [e]-Comparison between Methanol group and Standard Group of same row; [f]-Comparison between Residue group and Standard Group of same row

**Table 4: Total flavonoids content of Fractions**

| S. No. | Fraction | Total Flavonoid content (mg/g of Catechol ±SEM) |
|---|---|---|
| 1 | Ethyl acetate Fraction | 8.73±0.041[a] |
| 2 | Methanol Fraction | 7.45±0.021 |
| 3 | Residue Fraction | 8.62±0.025[c] |

Values are expressed as Mean ± SEM; (n = 3); Different Superscripted letters (a, b, c) means in the same column Means are significantly different at *P*< 0.05 (One Way ANOVA followed by Turkey – Kramer Multiple Comparison test); [a]-Comparison between Ethyl acetate group and Methanol Group; [b]-Comparison between Ethyl acetate group and Residue Group; [c]- Comparison between Methanol group and Residue Group

The Iron chelating activity of the fractions has been mentioned in table 3 and fig. 3. From the results it is evident that the $IC_{50}$ values are; 210.86µg/ml, 462.38µg/ml and 586.14µg/ml for Ethylacetate fraction, Methanol fraction and Residual fraction respectively. The $IC_{50}$ value for the standard EDTA is 90.99µg/ml.

Among the three fractions ethyl acetate is having the lowest $IC_{50}$ value (210.86 µg/ml) in the case of Iron chelating assay. If the results of all the three results are taken into account the ethylacetate fraction may be considered to be a better antioxidant in comparison to the other two fractions, whereas it is evident the fractions are showing more or less similar type of activity.

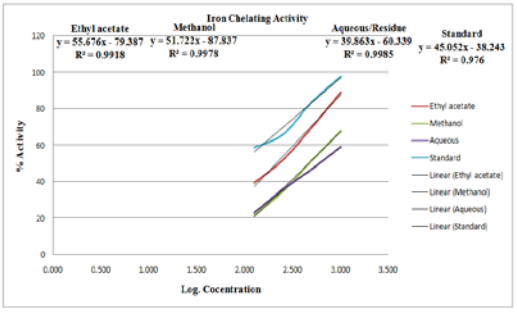


**Fig. 3: Iron-chelating activity of fractions**

**Table 5: Total Phenol content of fractions**

| S. No. | Fraction | Total phenol content (mg/g of Catechol±SEM) |
|---|---|---|
| 1 | Ethyl acetate Fraction | 8.43 ±0.051[a,b] |
| 2 | Methanol Fraction | 7.25 ±0.021[c] |
| 3 | Residue Fraction | 6.32 ±0.028 |

Values are expressed as Mean ± SEM; (n = 3); Different Superscripted letters (a, b, c) means in the same column Means are significantly different at *P*< 0.05 (One Way ANOVA followed by Turkey – Kramer Multiple Comparison test); [a]-Comparison between Ethyl acetate group and Methanol Group; [b]-Comparison between Ethyl acetate group and Residue Group; [c]- Comparison between Methanol group and Residue Group

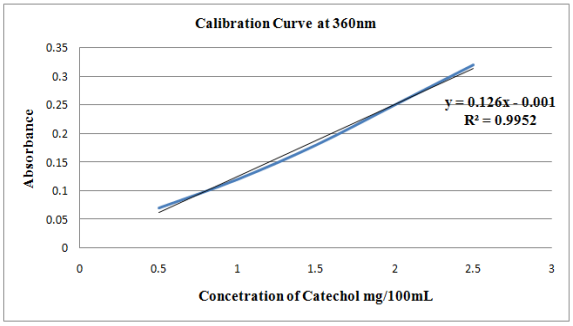


**Fig. 4: Total flavonoids content of fractions**

The estimation of total flavonoid contents of different fractions have been depicted in table 4 and fig. 4. The flavonoid content has been expressed as mg catechol equivalent per gm of dry weight of the fraction. The results reveal that the flavonoids content of the fractions are; 8.73, 7.45 and 8.62 with respect to Ethylacetate fraction, Methanol fraction and Residue fraction. The flavonoid content of ethylacetate fraction is significantly higher than methanol fraction where there is no difference between ethylacetate fraction and residual fraction with respect to flavonoid content.

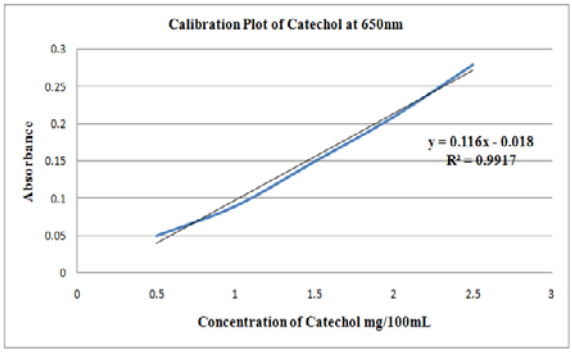


**Fig. 5: Total Phenol content of fractions**

The total phenol contents of different fractions have been depicted in table 5 and fig. 5. The phenol content has been expressed as mg catechol equivalent per gm of dry weight of the substance. The results reveal that the phenol content of the fractions are; 8.43, 7.25 and 6.32 with respect to ethylacetate fraction, Methanol fraction and Residue fraction. Ethylacetate fraction is having significantly high content of phenolic compounds in comparison to methanol fraction and residual fraction.

## DISCUSSION

Oxidative stress has been established to play a key role in the development of diabetes and antioxidants are being considered to have a role in the alleviation of diabetes [17]. Oxidative stress also plays a key role in development of diabetic complications. Production of highly reactive oxygen species which are toxic to the cell membrane interact with the lipidbilayer and produce lipid peroxides [18, 19, 20]. Streptozotocin (STZ) induced diabetes in animals involves the production of free radicals, where oxygen free radicals formed causes pancreatic injury and could be responsible for increased blood sugar seen in animals [21]. As our previous studies has revealed that the hydro-alcoholic extract of *Annona reticulata* leaves is having potential anti-hyperglycemic property [2], the role of antioxidant activity can't be ruled out for such property.

The results of Nitric Oxide Radical Scavenging assay reveals that; among the three fractions Methanol fraction and Residual fraction are having almost similar $IC_{50}$ value, i. e. 328.09 µg/ml and 329.61 µg/ml respectively. The $IC_{50}$ value of standard Ascorbate is 387.26 µg/ml and is higher than methanolic fraction and residual fraction. Among the three fractions methanolicfraction is having the lowest $IC_{50}$ value (328.09 µg/ml) in case of NO radical scavenging activity. In DPPH Assay model it has been observed that; the $IC_{50}$ value for free radical scavenging in DPPH model for Ethylacetate fraction is 87.50 µg/ml and is the lowest among all the fractions. The $IC_{50}$ value for ethylacetate is approximately two times lesser whencompared with standard Rutin ($IC_{50}$ is 39.54 µg/ml). The result of Iron chelating activity of the fractions suggests that; among the three fractions ethylacetate is having the lowest $IC_{50}$ value (210.86 µg/ml) in case of Iron chelating assay and approximately twice that of standard EDTA ($IC_{50}$ is 90.99µg/ml). If the results of all the three results are taken into account the ethylacetate fraction may be considered to be a better antioxidant in comparison to the other two fractions, though not significantly different. The similar type of observation has been done Abdulwali& co-workers while studying antidiabetic and antioxidant properties ofethanolic extract and different fractions of *Bruceajavanica* seed [22].

The result of the total Flavonoid content estimation of different fractions revealed that; Ethylacetate fraction (8.73) is having higher amount of flavonoids than methanol fraction (7.45) and residual fraction (8.62). The flavonoid content of ethylacetate fraction is significantly higher than methanol fraction. Evaluations of total phenolic content of the fractions revealed that ethylacetate fraction (8.43) is having significantly high content of phenolic compared to methanol fraction (7.25) and residue fraction (6.32). Flavonoid and phenols are the compounds known to have antioxidant activity and relationship between content of these compounds with antioxidant activity of natural products are well established [23]. The high content of flavonoids and phenolics in ethylacetate fraction supports the observed anti-oxidant property of ethylaceate fraction which has shown lower $IC_{50}$ value than the other two fractions in two (DPPH Assay and Iron Chelating Assay) out of the three studies conducted.

Natural antioxidant compounds like phenolics, phenylpropanoids and flavonoids prevent free radical chain reaction in biological systems and provide additional health benefits [24]. Flavonoids are known as excellent free radical scavenging agents and known to have properties for management of diabetes and it's complications [25, 26, 27]. Flavonoid also leads to the regeneration of pancreatic β-cells, reduces necrosis and degeneration and thus, may be effective in treating hyperglycemia thereby preventing diabetic complications [28]. Flavonoids are also known to decrease triglyceride level [29]. Sharma and co-workers have found fractions rich with flavonoid have both hypoglycemic and hypolipidemic effects while working on effect of *Eugenia jambolana* seeds on STZ induced rats [30].

Phenolics are known to be potent radical scavenger and inhibit free radical mediated formation of AGEs and counteract the diabetic complications. Phenolic compounds are also known to decrease blood glucose in STZ induced diabetic rats. Increase in insulin secretion due to regeneration of β-cells brings reduction in oxidative stress [31]. Phenolics tend to increase the GSH levels and thus decreases levels of lipid peroxidation in diabetic rats, and contribute in management of diabetes and it's complications [32]. So, plant products with higher content of flavonoids and phenols may be considered as products having potential to provide protection against diabetes and it's complications.

## CONCLUSION

From the results of the experiments carried out in this research work it may be suggested that the ethyl acetate fraction prepared from the hydro-alcoholic extract of *Annona reticulata* is having better antioxidant potential in comparison to methanol and residual fractions. The higher content of flavonoids and phenolics in ethyl acetate fraction supports the assumption. This may also be suggested that the components of the ethyl acetate fraction might be the major contributor towards the observed biological activity of the leaves of *Annona reticulata* Linn.

## CONFLICT OF INTERESTS

Declared None

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